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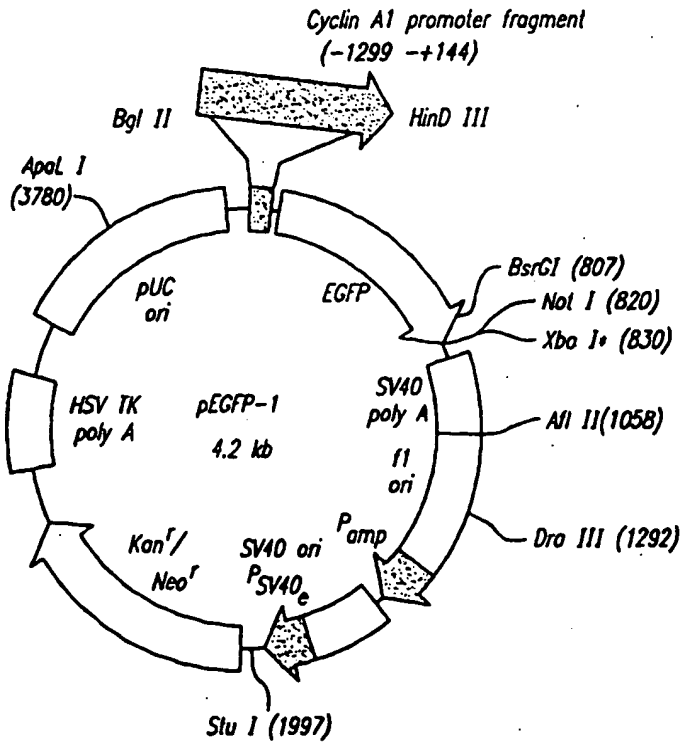
(54) Title: TRANSFECTION OF MALE GERM CELLS FOR GENERATION OF SELECTABLE TRANSGENIC STEM CELLS

(57) Abstract

Disclosed is a method of obtaining selectable transgenic stem cells of a vertebrate by transfecting a male germ cell with a transfection mixture comprising a nucleic acid construct containing a transcriptional unit of a stem cell-specific promoter, for example, a cyclin A1 promotor, operatively linked to a gene encoding a fluorescent or light-emitting reporter protein. The transfection mixture is a composition for transfection, in vivo or ex vivo, of a vertebrate's male germ cells, which comprises a nucleic acid or transgene, and a gene delivery system, and optionally a protective internalizing agent, such as an endosomal lytic agent, a virus or a viral component, which is internalized by cells along with the transgene and which enhances gene transfer through the cytoplasm to the nucleus of the male germ cell. In stem cells, other than germ cells, grown in vivo, expression of the reporter gene from a cyclin A1 promoter is facilitated by preventing methylation of promoter DNA by the use of flanking insulator elements in the nucleic acid construct. Alternatively, inhibitors of DNA methylation can be used in an in vitro growth medium. A kit contains components of the transfection mixture. Selectable transgenic stem cells have stably integrated the DNA, and non-human transgenic vertebrates comprise these selectable transgenic stem cells.



TRANSFECTION OF MALE GERM CELLS FOR GENERATION OF SELECTABLE TRANSGENIC STEM CELLS

BACKGROUND OF THE INVENTION

Throughout this application various publications are referenced within parentheses. The disclosures of these publications in their entireties are hereby incorporated by reference in this application in order to more fully describe the state of the art to which this invention pertains.

1. THE FIELD OF THE INVENTION

This invention relates to the medical arts, particularly to the field of transgenics and gene therapy. The invention is particularly directed to the field of transgenic vertebrate stem cells.

2. DISCUSSION OF THE RELATED ART

The field of transgenics was initially developed to understand the action of a single gene in the context of the whole animal and phenomena of gene activation, expression, and interaction. This technology has been used to produce models for various diseases in humans and other animals. Transgenic technology is among the most powerful tools available for the study of genetics, and the understanding of genetic mechanisms and function. It is also used to study the relationship between genes and diseases. About 5,000 diseases are caused by a single genetic defect. More commonly, other diseases are the result of complex interactions between one or more genes and environmental agents, such as viruses or carcinogens. The understanding of such interactions is of prime importance for the development of therapies, such as gene therapy and drug therapies, and also treatments such as organ transplantation. Such treatments compensate for functional deficiencies and/or may eliminate undesirable

is in contrast to only 10-20 eggs in a mouse even after treatment with superovulatory drugs. A similar situation is true for ovulation in nearly all larger animals. For this reason alone, male germ cells will be a better target for introducing foreign DNA into the germ line, leading to the generation of transgenic animals with increased efficiency and after simple, natural mating.

Spermatogenesis is the process by which a diploid spermatogonial stem cell provides daughter cells which undergo dramatic and distinct morphological changes to become self-propelling haploid cells (male gametes) capable, when fully mature, of fertilizing an ovum.

Primordial germ cells are first seen in the endodermal yolk sac epithelium at E8 and are thought to arise from the embryonic ectoderm (A. McLaren and Buehr, Cell Diff. Dev. 31:185 [1992]; Y. Matsui et al., Nature 353:750 [1991]). They migrate from the yolk sac epithelium through the hindgut endoderm to the genital ridges and proliferate through mitotic division to populate the testis.

At sexual maturity the spermatogonium goes through 5 or 6 mitotic divisions before it enters meiosis. The primitive spermatogonial stem cells (Ao/As) proliferate and form a population of intermediate spermatogonia types Apr, Aal, A1-4 after which they differentiate into type B spermatogonia. The type B spermatogonia differentiate to form primary spermatocytes which enter a prolonged meiotic prophase during which homologous chromosomes pair and recombine. The states of meiosis that are morphologically distinguishable are; preleptotene, leptotene, zygotene, pachytene, secondary spermatocytes and the haploid spermatids. Spermatids undergo great morphological changes during spermatogenesis, such as reshaping the nucleus, formation of the acrosome and assembly of the tail (A.R. Bellve *et al.*, *Recovery, capacitation, acrosome reaction, and fractionation of sperm*, Methods Enzymol. 225:113-36 [1993]). The spermatocytes and spermatids establish vital contacts with the Sertoli cells through unique hemi-junctional attachments with the Sertoli cell membrane. The final changes in the maturing spermatozoan take place in the genital tract of the female prior to fertilization.

Initially, attempts were made to produce transgenic animals by adding DNA to spermatozoa which were then used to fertilize mouse eggs in vitro. The fertilized eggs were then transferred to pseudopregnant foster females, and of the pups born, 30% were reported to be transgenic and express the transgene. Despite repeated efforts by others, however, this

a fluorochrome and detection of stem cells is by fluorescence activated cell sorter (FACS). Murray *et al.* taught a method of purifying a population of hematopoietic stem cells expressing a CDw109 marker that used binding of monoclonal antibodies specific for Cdw109. (L. Murray *et al.*, *Method of purifying a population of cells enriched for hematopoietic stem cells, populations of cells obtained thereby and methods of use thereof*, U.S. Pat. No. 5,665,557).

Transgenic neural stem cells (NSCs) have also been identified and selected using immunofluorescence or other immunostaining techniques. (J.D. Flax *et al.*, *Engraftable human neural stem cells respond to developmental cues, replace neurons, and express foreign genes*, Nature Biotechnol. 16(11):1033-39 [1998]; O. Bruestle *et al.*, *Chimeric brains generated by intraventricular transplantation of fetal human brain cells into embryonic rats*, Nature Biotechnol. 16(11):1040-44 [1998]).

However, such immunologically based methods as these have limited usefulness in identifying or selecting stem cells, because they rely on tissue- or lineage-specific epitopes and do not consistently leave the cells in a viable condition. Others have addressed the latter problem using non-lethal methods for labeling transgenic cells, particularly using genes encoding fluorescent or bioluminescent markers. For example, Chalfie *et al.* disclosed a recombinant DNA molecule comprising the green fluorescent protein gene operatively linked to any exogenous regulatory element. (M. Chalfie *et al.*, *Uses of green-fluorescent protein*, U.S. Pat. No. 5,491,084). Cormier *et al.* taught a recombinant DNA vector comprising the gene for apoaequorin, a bioluminescent protein. (M.J. Cormier *et al.*, *Recombinant DNA vectors capable of expressing apoaequorin*, USPN 5,422,266).

Contag *et al.* disclosed a method for detecting a transformed cell of interest expressing a light-generating moiety in vivo. (C.H. Contag, *Non-invasive localization of a light-emitting conjugate in a mammal*, U.S. Pat. No. 5,650,135). Similarly, Horan *et al.* disclosed a method for tracking cells in vivo related to labeling cells with a fluorecent cyanine dye. (P.K. Horan *et al.*, *In vivo cellular tracking*, U.S. Pat. No. 4,762,701). And Patterson *et al.* taught a method of detecting cells expressing a specific nucleotide target sequence by using fluorescently labeled complementary nucleic acid probes and fluorescence-activated flow cytomety (FACS). (Patterson *et al.*, *Method of detecting amplified nucleic sequences in cells by flow cytometry*, U.S. Pat. No. 5,840,478).

adenovirus E1A-associated protein p60 and behaves differently from cyclin B, Nature 346:760-63 [1990]; C. Desdouets *et al.*, *Cyclin A: function and expression during cell proliferation*, Prog. Cell Cycle Res. 1:15-23 [1995]). Cyclin A also forms a complex with CDC2, the activity of which peaks at the G_2 to M transition, and the kinase activity of cyclin A/CDC2 is also required for M-phase entry (M. Pagano *et al.* [1992]).

Two kinds of cyclin A were first found in *Xenopus*; early embryos contained both cyclin A1 and cyclin A2. Later in development, cyclin A2, which shares considerable homology to mammalian cyclin A2, was found throughout the embryo, whereas cyclin A1 was found only in the testis and ovary. (J.A. Howe *et al.*, *Identification of a developmental timer regulating the stability of embryonic cyclin A and a new somatic A-type cyclin at gastrulation*, Genes Dev. 9(10):164-76 [1995]). In the mouse, cyclin A2 was found in a number of tissues during development, but cyclin A1 expression was highly restricted, with high levels measured in late pachytene spermatocytes. (C. Sweeney *et al.*, *A distinct cyclin A is expressed in germ cells in the mouse*, Development 122(1):53-64 [1996]).

Cyclin A1 is not expressed in fully differentiated cells of non-embryonic tissues, but can be expressed in a wide variety of stem cells, including male and female germ cells, brain stem cells, hematopoietic progenitor cells, as well as in a majority of myeloid leukemic cells and undifferentiated hematological malignancies. (R. Yang *et al.*, *Characterization of a second human cyclin A that is highly expressed in testis and in several leukemic cell lines*, Cancer Res. 57(5):913-20 [1997]; A. Kramer *et al.*, *Cyclin A1 is predominantly expressed in hematological malignancies with myeloid differentiation*, Leukemia 12(6):893-98 [1998]; C. Sweeney *et al.* [1996]; J.A. Howe *et al.* [1995]). The pattern of cyclin A1 expression indicates that its regulation differs from that of cyclin A2, and this may be related to differential binding by cyclin A1 and cyclin A2 promoters of transcriptional initiation factors, such as the Sp1 family of initiation factors.

The Sp1 family of initiation factors is related to the regulation of differentiation in stem cells. (K.L. Block et al., Blood 88:2071-80 [1996]; H.M. Chen et al., J. Biol. Chem. 268:8230-39 [1993]; R.K. Margana et al., J. Biol. Chem. 272:3083-90 [1997]). Sp1 is expressed at high levels in tissues where cyclin A1 expression is found. (C. Sweeney *et al.* [1996]). Also, induction of Sp1 was found to be associated with differentiation of embryonal carcinoma cells and Sp1 was causally linked to expression of the fibronectin gene, providing

activator they may be important for the activation of cell cycle genes such as cyclin A1. (Reviews: S.A. Ness, Biochim Biophys. Acta 1288:F123-F139 [1996]; M.K. Saville and R.J. Watson, Adv. Cancer Res. 72:109-40 [1998]).

The present invention addresses the need for spermatogenic transfection, either in vitro or in vivo, that is highly effective in transferring allogeneic as well as xenogeneic genes into the animal's germ cells and in producing transgenic vertebrate animals. The present technology addresses the requirements of germ line and stem cell line gene therapies in humans and other vertebrate species. Further, the method of the present invention particularly addresses the problem of identifying and selecting stem cells from non-stem cells including differentiated somatic cells, especially from non-embryonic biological sources.

These and other benefits and features of the present invention are described herein.

SUMMARY OF THE INVENTION

The present invention relates to the in vivo and ex vivo (in vitro) transfection of eukaryotic animal germ cells with a desired genetic material. Briefly, the in vivo method involves injection of genetic material together with a suitable vector directly into the testicle of the animal. In this method, all or some of the male germ cells within the testicle are transfected in situ, under effective conditions. The ex vivo method involves extracting germ cells from the gonad of a suitable donor or from the animal's own gonad, using a novel isolation method, transfecting them in vitro, and then returning them to the testis under suitable conditions where they will spontaneously repopulate it. The ex vivo method has the advantage that the transfected germ cells may be screened by various means before being returned to the testis to ensure that the transgene is incorporated into the genome in a stable state. Moreover, after screening and cell sorting only enriched populations of germ cells may be returned. This approach provides a greater chance of transgenic progeny after mating.

This invention also relates to a novel method for the isolation of spermatogonia, comprising obtaining spermatogonia from a mixed population of testicular cells by extruding the cells from the seminiferous tubules and gentle enzymatic disaggregation. The spermatogonia or stem cells which are to be genetically modified, may be isolated from a mixed cell population by a novel method including the utilization of a promoter sequence, which is only active in stem cells, for example the cyclin A1 promoter, or in cycling

into the genome of the germ cells. Following incorporation of the DNA, the treated animal is either allowed to breed naturally, or reproduced with the aid of assisted reproductive technologies, and the progeny selected for the desired trait.

This technology is applicable to the production of transgenic animals for use as animal models, and to the modification of the genome of an animal, including a human, by addition, modification, or subtraction of genetic material, often resulting in phenotypic changes. The present methods are also applicable to altering the carrier status of an animal, including a human, where that individual is carrying a gene for a recessive or dominant gene disorder, or where the individual is prone to pass a multigenic disorder to his offspring.

A preparation suitable for use with the present methods comprises a polynucleotide segment encoding a desired trait and a transfection promotion agent, and optionally an uptake promotion agent which is sometime equipped with agents protective against DNA breakdown. The different components of the transfection composition (mixture) are also provided in the form of a kit, with the components described above in measured form in two or more separate containers. The kit generally contains the different components in separate containers and instructions for effective use. Other components may also be provided in the kit as well as a carrier.

Thus the present technology is of great value in the study of stem cells and cellular development, and in producing transgenic vertebrate animals as well as for repairing genetic defects. The present technology is also suitable for germ line and stem cell line gene therapy in humans and other vertebrate animal species. The present invention is also valuable in identifying cell lineages before full differentiation to facilitate modification and/or engineering of specific tissues in vitro for their subsequent transplantation in the treatment of disease or trauma.

BRIEF DESCRIPTION OF THE DRAWINGS

Figure 1 represents a map of DNA construct pCyclinA1-EGFP-1.

Figure 2 represents transcriptional start sites in the human cyclin A1 gene.

Figure 3 represents 5' upstream region of the human cyclin A1 gene.

Figure 4 represents transactivation activity of cyclin A1 promoter fragments in Hela cells.

may take place after biopsy of one or both gonads, or after examination of the animal's ejaculate amplified by the polymerase chain reaction to confirm the incorporation of the desired nucleic acid sequence. In order to simplify the confirmation of the actual incorporation of the desired nucleic acid, the initial transfection may include a co-transfected reporter gene, such as a gene encoding for Green Fluorescent Protein (or encoding enhanced Green Fluorescent Protein [EGFP]), Yellow Fluorescent Protein, Blue Fluorescent Protein, a phycobiliprotein, such as phycoerythrin or phycocyanin, or any other protein which fluoresces under a suitable wave-length of ultraviolet light.

Alternatively, male germ cells may be isolated from a donor animal and transfected, or genetically altered in vitro to impart the desired trait. Following this genetic manipulation, germ cells which exhibit any evidence that the DNA has been modified in the desired manner are selected, and transferred to the testis of a suitable recipient animal. Further selection may be attempted after biopsy of one or both gonads, or after examination of the animal's ejaculate amplified by the polymerase chain reaction to confirm whether the desired nucleic acid sequence was actually incorporated. As described above, the initial transfection may have included a co-transfected reporter gene, such as a gene encoding the Green Fluorescent Protein (or enhanced Green Fluorescent Protein [EGFP]), Yellow Fluorescent Protein, Blue Fluorescent Protein, a phycobiliprotein, such as phycoerythrin or phycocyanin, or any other protein which fluoresces under light of suitable wave-lengths. Before transfer of the germ cells, the recipient testis are generally treated in one, or a combination, of a number of ways to inactivate or destroy endogenous germ cells, including by gamma irradiation, by chemical treatment, by means of infectious agents such as viruses, or by autoimmune depletion or by combinations thereof. This treatment facilitates the colonization of the recipient testis by the altered donor cells.

Animals that were shown to carry suitably modified sperm cells then may be either allowed to mate naturally, or alternatively their spermatozoa are used for insemination or in vitro fertilization. The thus obtained transgenic progeny may be bred, whether by natural mating or artificial insemination, to obtain further transgenic progeny. The method of this invention has a lesser number of invasive procedures than other available methods, and a high rate of success in producing incorporation into the progeny's genome of the nucleic acid sequence encoding a desired trait.

pressure. The micropipette may be made of a suitable material, such as metal or glass, and is usually made from glass tubing which has been drawn to a fine bore at its working tip, e.g. using a pipette puller. The tip may be angulated in a convenient manner to facilitate its entry into the testicular tubule system. The micropipette may be also provided with a beveled working end to allow a better and less damaging penetration of the fine tubules at the injection site. This bevel may be produced by means of a specially manufactured grinding apparatus. The diameter of the tip of the pipette for the in vivo method of injection may be about 15 to 45 microns, although other sizes may be utilized as needed, depending on the animal's size. The tip of the pipette may be introduced into the rete testis or the tubule system of the testicle, with the aid of a binocular microscope with coaxial illumination, with care taken not to damage the wall of the tubule opposite the injection point, and keeping trauma to a minimum. On average, a magnification of about x25 to x80 is suitable, and bench mounted micromanipulators are not severally required as the procedure may be carried out by a skilled artisan without additional aids. A small amount of a suitable, non-toxic dye, may be added to the gene delivery fluid to confirm delivery and dissemination to the tubules of the testis. It may include a dilute solution of a suitable, non-toxic dye, which may be visualized and tracked under the microscope.

In this manner, the gene delivery mixture is brought into intimate contact with the germ cells. The gene delivery mixture typically comprises the modified nucleic acid encoding the desired trait, together with a suitable promoter sequence, and optionally agents which increase the uptake of the nucleic acid sequence, such as liposomes, retroviral vectors, adenoviral vectors, adenovirus enhanced gene delivery systems, or combinations thereof. A reporter construct such as the gene encoding for Green Fluorescent Protein may further be added to the gene delivery mixture. Targeting molecules such as c-kit ligand may be added to the gene delivery mixture to enhance the transfer of the male germ cell.

For the ex vivo (in vitro) method of genetic alteration, the introduction of the modified germ cells into the recipient testis may be accomplished by direct injection using a suitable micropipette. Support cells, such as Leydig or Sertoli cells that provide hormonal stimulus to spermatogonial differentiation, may be transferred to a recipient testis along with the modified germ cells. These transferred support cells may be unmodified, or, alternatively, may themselves have been transfected, together with- or separately from the germ cells.

by receptors on the cell surface of the germ cell, such as the c-kit ligand or modifications thereof.

Other preferred transfecting agents include lipofectin, lipfectamine, DIMRIE C, Superfect, and Effectin (Qiagen). Although these are not as efficient transfecting agents as viral transfecting agents, they have the advantage that they facilitate stable integration of xenogeneic DNA sequence into the vertebrate genome, without size restrictions commonly associated with virus-derived transfecting agents.

"Virus", as used herein, means any virus, or transfecting fragment thereof, which may facilitate the delivery of the genetic material into male germ cells. Examples of viruses which are suitable for use herein are adenoviruses, adeno-associated viruses, retroviruses such as human immune-deficiency virus, lentiviruses, such as Moloney murine leukemia virus and the retrovirus vector derived from Moloney virus called vesicular-stomatitis-virus-glycoprotein (VSV-G)-Moloney murine leukemia virus, mumps virus, and transfecting fragments of any of these viruses, and other viral DNA segments that facilitate the uptake of the desired DNA segment by, and release into, the cytoplasm of germ cells and mixtures thereof. The mumps virus is particularly suited because of its affinity for immature sperm cells including spermatogonia. All of the above viruses may require modification to render them non-pathogenic or less antigenic. Other known vector systems, however, may also be utilized within the confines of the invention.

"Genetic material", as used herein, means DNA sequences capable of imparting novel genetic modification(s), or biologically functional characteristic(s) to the recipient animal. The novel genetic modification(s) or characteristic(s) may be encoded by one or more genes or gene segments, or may be caused by removal or mutation of one or more genes, and may additionally contain regulatory sequences. The transfected genetic material is preferably functional, that is it expresses a desired trait by means of a product or by suppressing the production of another. Examples of other mechanisms by which a gene's function may be expressed are genomic imprinting, i.e. inactivation of one of a pair of genes (alleles) during very early embryonic development, or inactivation of genetic material by mutation or deletion of gene sequences, or by repression of a dominant negative gene product, among others.

In addition, novel genetic modification(s) may be artificially induced mutations or variations, or natural allelic mutations or variations of a gene(s). Mutations or variations may

+538), with transcribed regions being underlined and the translational start site at nt. +127 to +129 being bolded:

-1299 TCGATCTGAT TTAGAGATTT AGGGATGGAT GTTTTAAAAA AAGCAAAAGT
-1249 AGTAACAGAC TATAGCATTG GTAATGTGTG TGTGCATATA TACATATTAT
-1199 TTTTAAAAAA ATAAAGTTCG ATTATTTCAC CTGGCTTGTC AGTCACCTAT
-1149 GCAGGCGTCT GAGCCCCCGG GTTTCCAGGA GCCCCCCGTA TAAGGACCCC
-1099 AGGGACTCCT CTCCCCACGC GGCCGGGCCG CCCGCCCGGC CCCCAGCCCG
-1049 GAGAGCTGCC ACCGACCCCC TCAACGTCCC AAGCCCCAGC TCTGTCGCCC
-0999 GCGTTCCTTC CTCTTCCTGG GCCACAATCT TGGCTTTCCC GGGCCGGCTT
-0949 CACGCAGTTG CGCAGGAGCC CGCGGGGGAA GACCTCTCGTGGGGACCTCG
-0899 AGCACGACGT GCGACCCTAA ATCCCCACAT CTCCTCTGCC GCCTCGCAGG
-0849 CCACATGCAC CGGGAGCCGG GCGGGGCAGG CGCGGCCCGC AAGGACCCCC
-0799 GCGATGGAGA CGCAACACTG CCGCGACTGC ACTTGGGGCA GCCCCGCCGC
-0749 GTCCCAGCCG CCTCCCGGCA GGAAGCGTAG GTGTGTGAGC CGACCCGGAG
-0699 CGAGCCGCGC CCTCGGGCCA GCGTGGGCAG GGCGCCGCAG CCTGCGCAGC
-0649 CCCGAGGACC CCGCGTCGCT CTCCCGAGCC AGGGTTCTCA GGAGCGGGCC
-0599 GCGCAGGAGA CGTTAGAGGG GGTTGTTAGC GGCTGTTGGG AGAACGGGTC
-0549 ACGGAAACAG TCCCTTCCAA AGCCGGGGCC ATCGTGGGGT GGGCGAGTCC
-0499 GCCCTCCCAG GCCGGGGGCG CGGACCAGAG GGGACGTGTG CAGACGGCCG
-0449 CGGTCAGCCC CACCTCGCCC GGGCGGAGAC GCACAGCTGG AGCTGGAGGG
-0399 CCGTCGCCCG TTGGGCCCTC AGGGGCCTGA ACGCCCAGGG GTCGCGGCGA
-0349 GTCCACCCGG AGCGAGTCAG GTGAGCAGGT CGCCATGGCG ATGCGGCCCC
-0299 GGAGAGCGCA CGCCTGCCGC GGTCGGCATG GAAACGCTCC CGCTAGGTCC
-0249 GGGGGCGCCG CTGATTGGCC GATTCAACAG ACGCGGGTGG GCAGCTCAGC
-0199 CGCATCGCTA AGCCCGGCCG CCTCCCAGGC TGGAATCCCT CGACACTTGG
-0149 TCCTTCCCGC CCCGCCCTTC CGTGCCCTGC CCTTCCCTGC CCTTCCCCGC
-0099 CCTGCCCCGC CCGGCCCGGC CCGGCCCTGC CCAACCCTGC CCCGCCCTGC
-0049 CCCGCCCAGC CGGCCACCTC TTAACCGCGA TCCTCCAGTG CACTTGCCAG
+0003 TTGTTCCGGA CACATAGAAA GATAACGACG GGAAGACGGG GCCCCGTTTG
+0053 GGGTCCAGGC AGGTTTTGGG GCCTCCTGTC TGGTGGGAGG AGGCCGCAGC
+0103 GCAGCACCCT GCTCGTCACT TGGGATGGAG ACCGGCTTTC CCGCAATCAT
+0153 GTACCCTGGA TCTTTTAT TG GGGGCTGGGG AGAAGAGTAT CTCAGCTGGG
+0203 AAGGACCGGG GCTCCCAGAT TTCGTCTTCC AGGTAACGTG GGTTTAGTAT

SEQ. ID. NO:2, or of any operative fragment of SEQ. ID. NO.:2 or non-human homologue thereof, in which the codon of the first translational start site is changed to another codon sequence, other than ATT, that is also not recognized as a translational start site: another preferred cyclin A1 promoter is a derivative of SEQ. ID. NO.:2 with the codon of the first translational start site deleted altogether. Other operative derivatives include cyclin A1 promoter sequences containing a mutation, polymorphism, or variant allele with respect to any nucleotide position of SEQ. ID. NO.:2 that does not eliminate promoter activity.

Similar to promoters in other cell cycle regulatory genes (B. Henglein *et al.*, Proc. Natl. Acad. Sci. (USA) 91:5490-94 [1994]; A. Hwang *et al.*, J. Biol. Chem. 270:28419-24 [1995]; E.W. Lam *et al.*, Oncogene 7:1885-90 [1992]), the cyclin A1 promoter does not possess a TATA-box motif. The nucleotides surrounding the transcriptional start site are likely to function as an initiator. The cyclin A1 promoter region contains multiple binding sites for transcription factor including GC boxes, Myb, and E2F sites.

The upstream region contains a GC rich region with multiple Sp1 binding sites that are essential for transcription from the cyclin A1 promoter. In contrast, predicted GC boxes in the cyclin A2 promoter are located more than 120 bp upstream of the transcriptional start site and these have not been shown to be essential for gene expression. GC boxes and the Sp1 family transcription factors are important in the regulation of expression from the cyclin A1 promoter. Six GC boxes are found in the first 200 bp upstream of the transcription start site. Omitting the four GC boxes between -112 and -37 almost completely abrogates promoter activity. Among GC boxes Nos. 1-4, the two closest to the transcriptional start sites are most critical. Of GC boxes Nos. 3 and 4, only one of these is necessary for a basal level of transcriptional activity of the promoter.

Sp1, the main activating factor of the Sp1 family, and Sp3 can bind to GC boxes Nos. 1 + 2 and 3 + 4. Analysis of these fragments in insect cells demonstrates that Sp1 reconstitutes cyclin A1 promoter activity in all fragments that involve the GC boxes Nos. 1-4. Sp1 (or at least a member of the Sp1 family) is required for cyclin A1 promoter activity through interaction with elements located between -112 and -37. Repression is likely to be accomplished by Sp3 and an as yet unidentified repressor mechanism that does not depend on E2F, CDE or CHR elements.

The DNA of animal cells is subject to methylation at the 5' carbon position of the

and thus expression of the reporter gene occurs within stem cell types other than germ cells. (M.J. Pikaart *et al.*, *Loss of transcriptional activity of a transgene is accompanied by DNA methylation and histone deacetylation and is prevented by insulators*, Genes Dev. 12:2852-62 [1998]; Chung *et al.*, *DNA sequence which acts as a chromatin insulator element to protect expressed genes from cis-acting regulatory sequences in mammalian cells*, U.S. Patent No. 5,610,053).

Alternatively, when the method of obtaining selectable transgenic stem cells is practiced to select stem cells grown in vitro, inhibitors of histone deacetylation and DNA methylation, such as trichostatin A or sodium butyrate, can be included in the culture medium to prevent silencing of reporter expression from the cyclin A1 promoter in a wide variety of cultured stem cells. (M.J. Pikaart *et al.* [1998]).

Suppression of methylation of the cyclin A1 promoter sequence can sometimes cause expression from a cyclin A1 promoter in kidney podocytes or in B-cells. Consequently, in applications in which selectable kidney stem cells are of interest, in accordance with the present method of obtaining selectable transgenic stem cells, fluorescent or luminescent podocytes that express a reporter gene from a cyclin A1 promoter are easily distinguished from fluorescing or light-emitting transgenic kidney stem cells by the distinct podocyte morphology (including protruding pedicels). In applications in which hematopoietic stem cells are of interest, fluorescent or luminescent B-cells are distinguished from transgenic hematopoietic stem cells by additionally using a B-cell-specific antibody conjugated to a fluorescent label that fluoresces or emits at a different wavelength from that of the reporter protein expressed as a result of cyclin A1-promoted transcription. For example, phycoerythricin-conjugated monoclonal antibodies against B-cell-specific surface epitopes can be applied to a cell population sample from bone marrow to distinguish B-cells from transgenic hematopoietic stem cells.

Three potential binding sites for Myb proteins are present within 100 bp of the transcription start sites of the cyclin A1 gene, located starting at -66, -27, and +2. (Fig. 3). Binding of c-myb protein occurs at the sites starting at -27 and +2, and c-myb protein transactivates expression from the human cyclin A1 promoter, as described in Example 23. In contrast, no consensus myb sites have been found for either the murine or human cyclin A2 promoter (X. Huet *et al.*, Molecular & Cellular Biology 16:3789-98 [1996]).

stem cells. And the present invention is also directed to a selectable transgenic stem cell, of any type, obtained by the method.

Preferred reporter genes encode fluorescent proteins including Green Fluorescent Protein (or EGFP), Yellow Fluorescent Protein, Blue Fluorescent Protein, a phycobiliprotein, such as phycoerythrin or phycocyanin, or any other protein which fluoresces under suitable wave-lengths of ultra-violet or other light. Another reporter gene suitable for some applications is a gene encoding a protein that can enzymatically lead to the emission of light from a substrate(s); for purposes of the present invention, such a protein is a "light-emitting protein." For example, a light-emitting protein includes proteins such as luciferase or apoaequorin.

In particular applications involving a transfected cell that expresses additional xenogeneic genes from any promoter, this expression may be linked to a reporter gene that encodes a different fluorescent or light-emitting protein from the reporter gene linked to the cyclin A1 promoter. Thus, multiple reporters fluorescing or emitting at different wavelengths can be chosen and cell selections based on the expression of multiple traits can be made. The selectable transgenic stem cells may be sorted, isolated or selected from non-stem cells with the aid of, for example, a FACS scanner set at the appropriate wavelength(s). Alternatively, they are isolated or selected manually from non-stem cells using conventional microscopic technology. It is an advantage of the present method of obtaining selectable transgenic stem cells that it allows stem cells to be selected or isolated from non-embryonic tissue.

The invention also relates to a nucleic acid construct comprising a human cyclin A1 promoter sequence in accordance with the present invention, or an operative fragment thereof. In a preferred embodiment for use in the method of obtaining a selectable transgenic stem cell, the cyclin A1 promoter is operatively linked to a DNA having a nucleotide sequence encoding a fluorescent protein or a light emitting protein. Other preferred embodiments employ a xenogeneic nucleic acid encoding any desired product or trait. For purposes of the present invention, "operatively linked" means that the promoter sequence, is located directly upstream from the coding sequence and that both sequences are oriented in a 5' to 3' manner, such that transcription could take place in vitro in the presence of all essential enzymes, transcription factors, co-factors, activators, and reactants, under favorable physical conditions, e.g., suitable pH and temperature. This does not mean that, in any particular cell, conditions will favor

phycoerythrin or phycocyanin, or any other protein which fluoresces under suitable wavelengths of light. These unique promoter sequences drive the expression of the reporter construct only in the cycling spermatogonia. The spermatogonia, thus, are the only cells in the mixed population which will express the reporter construct(s) and they, thus, may be isolated on this basis. In the case of a fluorescent reporter construct, the cells may be sorted with the aid of, for example, a FACS set at the appropriate wavelength(s) or they may be selected by chemical methods.

The method of the invention is suitable for application to a variety of vertebrate animals, all of which are capable of producing gametes, i.e. sperm or ova. Thus, in accordance with the invention novel genetic modification(s) and/or characteristic(s) may be imparted to animals, including mammals, such as humans, non-human primates, for example simians, marmosets, domestic agricultural (farm) animals such as sheep, cows, pigs, horses, particularly race horses, marine mammals, feral animals, felines, canines, pachyderms, rodents such as mice and rats, gerbils, hamsters, rabbits, and the like. Other animals include fowl such as chickens, turkeys, ducks, ostriches, emus, geese, guinea fowl, doves, quail, rare and ornamental birds, and the like. Of particular interest are endangered species of wild animal, such rhinoceros, tigers, cheetahs, certain species of condor, and the like.

The present invention is also related to a transgenic non-human vertebrate comprising a selectable transgenic stem cell in accordance with the present invention. Broadly speaking, a "transgenic" vertebrate animal is one that has had foreign DNA permanently introduced into its cells. The foreign gene(s) which (have) been introduced into the animal's cells is (are) called a "transgene(s)". The present invention is applicable to the production of transgenic animals containing xenogeneic, i.e., exogenous, transgenic genetic material, or material from a different species, including biologically functional genetic material, in its native, undisturbed form in which it is present in the animal's germ cells. In other instances, the genetic material is "allogeneic" genetic material, obtained from different strains of the same species, for example, from animals having a "normal" form of a gene, or a desirable allele thereof. Also the gene may be a hybrid construct consisting of promoter DNA sequences and DNA coding sequences linked together. These sequences may be obtained from different species or DNA sequences from the same species that are not normally juxtaposed. The DNA

albumin and a modified DMEM medium. The cells may be incubated in the enzyme mixture for a period of about 5 min to about 30 min, more preferably about 15 to about 20 min, at a temperature of about 33°C to about 37°C, more preferably about 36 to 37°C. After washing the cells free of the enzyme mixture, they may be placed in an incubation medium such as DMEM, and the like, and plated on a culture dish. Any of a number of commercially available transfection mixtures may be admixed with the polynucleotide encoding a desire trait or product for transfection of the cells. The transfection mixture may then be admixed with the cells and allowed to interact for a period of about 2 hrs to about 16 hrs, preferably about 3 to 4 hrs, at a temperature of about 33°C to about 37°C, preferably about 36°C to 37°C, and more preferably in a constant and/or controlled atmosphere. After this period, the cells are preferably placed at a lower temperature of about 33°C to about 34°C, preferably about 30-35°C for a period of about 4 hrs to about 20 hrs, preferably about 16 to 18 hrs. Other conditions which do not deviate radically from the ones described may also be utilized as an artisan would know.

The present method is applicable to the field of gene therapy, since it permits the introduction of genetic material encoding and regulating specific genetic traits. Thus, in the human, for example, by treating parents it is possible to correct many single gene disorders which otherwise might affect their children. It is similarly possible to alter the expression of fully inheritable disorders or those disorders having at least a partially inherited basis, which are caused by interaction of more than one gene, or those which are more prevalent because of the contribution of multiple genes. This technology may also be applied in a similar way to correct disorders in animals other than human primates. In some instances, it may be necessary to introduce one or more "gene(s)" into the germ cells of the animal to attain a desired therapeutic effect, as in the case where multiple genes are involved in the expression or suppression of a defined trait. In the human, examples of multigenic disorders include diabetes mellitus caused by deficient production of, or response to, insulin, inflammatory bowel disease, certain forms of atheromatus cardiovascular disease and hypertension, schizophrenia and some forms of chronic depressive disorders, among others. In some cases, one gene may encode an expressible product, whereas another gene encodes a regulatory function, as is known in the art. Other examples are those where homologous recombinant methods are applied to repair point mutations or deletions in the genome, inactivation of a

humans or children, undergoing oncological treatments for such diseases such as leukemia or Hodgkin's lymphoma. These treatments frequently irreversibly damage the testicle and, thus, render it unable to recommence spermatogenesis after therapy by, for example, irradiation or chemotherapy. The storage of germ cells and subsequent testicular transfer allows the restoration of fertility. In such circumstances, the transfer and manipulation of germ cells as taught in this invention are accomplished, but transfection is generally not relevant or needed.

In species other than humans, the present techniques are valuable for transport of gametes as frozen germ cells. Such transport will facilitate the establishment of various valued livestock or fowl, at a remote distance from the donor animal. This approach is also applicable to the preservation of endangered species across the globe.

The method of obtaining selectable transgenic stem cells, the selectable transgenic stem cells, the transgenic non-human vertebrates and vertebrate semen, and the nucleic acid contructs and kits, in accordance with the present invention, are valuable tools in the study of cellular differentiation and development and in developing new therapies for diseases related to cell differentiation, such as cancer, or for the regeneration of tissues after traumatic injuries. The present invention is valuable in identifying cell lineages before full differentiation to facilitate modification and/or engineering of specific tissues in vitro for their subsequent transplantation in the treatment of disease or trauma. It is an advantage of the present method of obtaining selectable transgenic stem cells that it allows stem cells to be selected or isolated from non-embryonic tissue, thus avoiding potential ethical and legal problems associated with the use of embryonic tissue. It is a further advantage that in accordance with the present invention, selectable transgenic stem cells can be selected and analyzed whether grown in vivo (i.e., in the whole organism) or in vitro.

The invention will now be described in greater detail by reference to the following non-limiting examples. The pertinent portions of the contents of all references, and published patent applications cited throughout this patent necessary for enablement purposes are hereby incorporated by reference.

EXAMPLES

In Vivo and In Vitro Adenovirus-enhanced Transferrin-polylysine-mediated Delivery

Example 3: Preparation of Adenoviral Particles

Adenovirus dl312, a replication-incompetent strain deleted in the Ela region, was propagated in the Ela trans-complementing cell line 293 as described by Jones and Shenk (Jones and Shenk, PNAS USA (1979) 79: 3665-3669). A large scale preparation of the virus was made using the method of Mittereder and Trapnell (Mittereder et al., "Evaluation of the concentration and bioactivity of adenovirus vectors for gene therapy", J. Urology, 70: 7498-7509 (1996)). The virion concentration was determined by UV spectroscopy, 1 absorbance unit being equivalent to 10 viral particles /ml. The purified virus was stored at -70°C.

Example 4: Formation of Transferrin-poly-L Lysine-DNA-Viral Complexes

6 μg transferrin-polylysine complex from Example 1 were mixed in 7.3 x 10^7 adenovirus d1312 particles prepared as in Example 3, and then mixed with 5 ug of the Green Lantern DNA construct of Example 2, and allowed to stand at room temperature for 1 hour. About 100 ul of the mixture were drawn up into a micropipette, drawn on a pipette puller, and slightly bent on a microforge. The filled micropipette was then attached to a picopump (Eppendorf), and the DNA complexes were delivered under continuous pressure, in vivo to mice as described in Example 6.

Controls were run following the same procedure, but omitting the transferrin-poly-lysine-DNA-viral complexes from the administered mixture.

Example 5: Comparison of Adenovirus-enhanced Transferrin-polylysine & Lipofectin Mediated Transfection Efficiency

The conjugated adenovirus particle complexed with DNA were tested on CHO cells in vitro prior to in vivo testing. For these experiments a luciferase reporter gene was used due to the ease of quantifying luciferase activity. The expression construct consists of a reporter gene encoding luciferase, is driven by the CMV promoter (Invitrogen, Carlsbad, CA 92008). CHO cells were grown in Dulbecco's modified Eagle's medium (DMEM) with 10% fetal calf serum. For gene transfer experiments CHO cells were seeded into 6 cm tissue culture plates and grown to about 50% confluency ($5x10^5$ cells). Prior to transfection the medium was aspirated and replaced with serum free DMEM. Cells were either transfected with transferrin-polylysine-DNA complexes or with lipofectin DNA aggregates. For the transferrin-

Wagner *et al.* (Schmidt *et al.*, Cell 4: 41-51 (1986); Wagner, E., *et al.* PNAS (1990), (USA) 81: 3410-3414 (1990)). In addition, this delivery system relies on the capacity of adenoviruses to disrupt cell vesicles, such as endosomes and release the contents entrapped therein. The transfection efficiency of this system is almost 2,000 fold higher than lipofection.

The male mice were anesthetized with 2% Avertin (100% Avertin comprises 10 g 2,2,2-tribromoethanol (Aldrich) and 10 ml t-amyl alcohol (Sigma), and a small incision made in their skin and body wall, on the ventral side of the body at the level of the hind leg. The animal's testis was pulled out through the opening by grasping at the testis fat pad with forceps, and the vas efferens tubules exposed and supported by a glass syringe. The EGFP DNA-transferrin-polylysine viral complexes were injected into a single vasa efferentia using a glass micropipette attached to a hand held glass syringe or a pressurized automatic pipettor (Eppendorf), and Trypan blue added to visualize the entry of the mixture into the seminiferous tubules. The testes were then placed back in the body cavity, the body wall was sutured, the skin closed with wound clips, and the animal allowed to recover on a warm pad.

Example 7: Detection of DNA and Transcribed Message

Nine (9) days after delivery of the genetic material to the animals' testis, two of the animals were sacrificed, their testes removed, cut in half, and frozen in liquid nitrogen. The DNA from one half of the tissues, and the RNA from the other half of the tissues were extracted and analyzed.

(a) Detection of DNA

The presence of DNA encoding enhanced green fluorescent protein (EGFP DNA) in the extracts was tested 9 days after administration of the transfection mixture using the polymerase chain reaction, and EGFP specific oligonucleotides. EGFP DNA was present in the testes of the animals that had received the DNA complexes, but was absent from sham operated animals.

(b) Detection of RNA

Example 10: In Vitro Transfection of Testicular Cells

Cells were isolated from the testes of three 10-day-old mice. The testes were decapsulated and the seminiferous tubules were teased apart and minced with sterile needles. The cells were incubated in enzyme mixture for 20 minutes at 37°C. The enzyme mixture was made up of 10 mg bovine serum albumin (embryo tested), 50 mg bovine pancreatic trypsin type III, Clostridium collagenase type I, 1 mg bovine pancreatic DNAse type I in 10 mls of modified HTF medium (Irvine Scientific, Irvine, CA). The enzymes were obtained from Sigma Company (St. Louis, Missouri 63178). After digestion, the cells were washed twice by centrifugation at 500 x g with HTF medium and resuspended in 250μl HTF medium. The cells were counted, and 0.5 x 10^6 cells were plated in a 60mm culture dish in a total volume of 5ml DMEM (Gibco-BRL, Life Technologies, Gaithesburg, MD 20884). A transfection mixture was prepared by mixing 5μg Green Lantern DNA (Gibco-BRL, Life Technologies, Gaithesburg, MD 20884) with 20μl Superfect (Qiagen, Santa Clarita, CA 91355) and 150μl DMEM. The transfection mix was added to the cells and they were allowed to incubate for 3 hours at 37°C, 5% CO_2 The cells were transferred to a 33 C incubator and incubated overnight.

The following morning the cells were assessed for transfection efficiency by counting the number of fluorescent cells. In this experiment the transfection efficiency was 90% (Figure not shown). The testicular cells transfected with Green Lantern viewed with Nomaski optics x20 show the same cells viewed with FITC. Nearly all the cells were fluorescent, which is confirmation of their successful transfection.

The cells were injected into the testis via the vasa efferentia using a micropipette. 3 x 10^5 cells in a total volume of 50μl were used for the injection. The cells were mixed with Trypan blue prior to the injection. Three adult mice were injected with transfected cells. The Balb/cByJ recipient mice had been irradiated 6 weeks prior to the injection with 800 Rads of gamma irradiation. One mouse became sick and was sacrificed 48 hours after the injection. The testes from this mouse were dissected, fixed and processed for histology.

The two remaining males were bred with normal females as shown. After 4 months pups were born. Litters are currently being screened for the integration of the transgene.

Example 11: Preparation of a Cell Suspension from Testicular Tissue for Cryopreservation

cloning vector. The construct was further digested using *Sma* I; and three fragments were subcloned into PUC19. The fragments were sequenced in both directions using cycle sequencing and an automated sequencer (ABI373) or Sequenase 2.0 (Amersham). The positions and lengths of the introns were determined by PCR amplification of the entire cyclin A1 coding region with different primers. Subsequently, PCR products were either subcloned using pGEM-T-Easy (Promega) or directly sequenced using cycle sequencing. Boundaries of the ~4.5 kb intron 2 were determined by direct sequencing of the lambda phage clone.

Generation of cyclin A1-luciferase DNA constructs. The initial luciferase constructs were generated by PCR amplification of the pRS316 plasmid containing the 2.2 kb cyclin A1 fragment. A *Bgl*II site at the 5' end and a *Bam* HI site at the 3' end were introduced and the Pfu amplified fragment was cloned into the *Bgl*II site of PGL3-Basic. The +144 fragment was generated to include the potential E2F site starting at +139. (Figure 3). The ATG in the primer (the initiating codon for cyclin A1 at nt. +127 to +129) was mutated to ATT to avoid the initiation of translation. All constructs were confirmed to have the correct sequence by DNA sequencing. The 5' deletions were generated by exonuclease III treatment using Kpn I/Sac I digested PGL3-Basic containing the -1299 to +144 fragment and the Erase-a-base kit (Promega). The endpoints of the deletions were determined by sequencing. The -37 fragment was constructed by digesting the -190 to +144 containing PGL3-Basic with *Nae*I and *Hind* III and subsequent cloning of the 200 bp fragment into PGL3-Basic digested with *Sma* I and *Hind* III.

Cell culture and transfection. Hela cells were cultured in DMEM medium supplemented with 10% fetal calf serum (FCS) containing 100 U/ml Penicillin and 100 μg/mL Streptomycin. For transfection, 5 x 10^5 cells were seeded into 60 mm plates 16 hours before transfection. Transfection was carried out using lipofectAMINE (Gibco, Life Technology) according to the manufacturer's protocol. Two μg of luciferase reporter plasmid was transfected together with 300 ng of a CMV-β-gal expression vector used for standardization. Cells were harvested and assayed for luciferase and β-galactosidase activity after 48 hours. All experiments were carried out in duplicate and were independently performed at least 3 times. Data of luciferase assays are shown as mean ± SEM of three independent experiments unless stated otherwise.

phenol-chloroform extracted, precipitated using NH_4^+ acetate and finally cloned into pGEM-T-Easy and sequenced.

The primer extension assay was carried out by reverse transcription of 10μg RNA (U937) using a ^{32}P-labeled primer 5'-CTC CTC CCA CCA GAC AGG A (SEQ. ID. NO.:17) corresponding to +95 to +76 on the cDNA. Hybridization was carried out overnight at 58°C. Superscript II was used for reverse transcription at 42°C for 50 minutes. Extension products were resolved on a 8% sequencing gel with a sequencing reaction being run in parallel. As negative controls, we used 10μg of t-RNA and a sample without RNA.

Electrophoretic Mobility Shift Assays. Nuclear extracts from Hela cells were prepared as described (A.M. Chumakov *et al.*, Oncogene 8:3005-11 [1993]). For gel retardation experiments, 1 ng of ^{32}P- labeled double stranded oligonucleotides containing either GC boxes 1+2 (5'-CCT GCC CCG CCC TGC CCC GCC CAG CC; SEQ. ID. NO.:18) or GC boxes 3+4 (5'-CCT TCC CCG CCC TGC CCC GCC CGG CCC; SEQ. ID. NO.:19) were incubated for 20 min at room temperature with 5 μg of Hela nuclear extract. The final reaction contained: 10 mM Tris-HCL, pH 7.5, 5% glycerol, 1 mM $MgCl_2$, 0.5 mM EDTA, 0.5 mM DTT, 100 mM NaCl and 1μg poly(dI-dC)-poly(dI-dC). For competition experiments, 100 ng of double stranded oligonucleotide containing either a Spl consensus site (5'-ATT CGA TCG GGG CGG GGC GAG C; SEQ. ID. NO.:20), the oligonucleotide used for gel retardation (see above) or a non-specific oligonucleotide (5'-GAG ACC GGC TCG AAC GCA ATC ATG T; SEQ. ID. NO.:21) were preincubated for 15 min at room temperature with the nuclear extracts before the addition of the labeled oligonucleotide. For supershift experiments, 2-3 μg of polyclonal antibody against Spl (Pep2, Santa Cruz) or Sp3 (D20, Santa Cruz) were preincubated with the nuclear extracts. Reactions were loaded on a 0.5x TBE /4% non-denaturing polyacrylamide gel and run for 2-3 h at 10 V/cm. Gels were dried and autoradiographed.

Site directed mutagenesis. Site directed mutagenesis was performed according to the method from Deng and Nickoloff (W.P. Deng and J.A. Nickoloff, Analyt. Biochem.200:81-88 [1992]) using the Transformer site directed mutagenesis kit (Clontech). In brief, phosphorylated oligonucleotides containing the desired mutation were annealed on the single-stranded PGL3-

14.5 kb insert contained the entire gene. A 2.2 kb fragment at the 5' end of the gene was subcloned and sequenced. The 2.2 kb fragment contained the first intron and parts of exon 2. The other exon-intron boundaries were analyzed by PCR-amplification and sequencing using sets of primers that span the entire coding region. The human cyclin A1 gene consists of 9 exons and 8 introns which extend over ~13 kb.

Example 14: Analysis of transcription start sites.

Transcription start sites were determined using primer extension analysis and 5' RACE. Primer extension was carried out as outlined in Example 12. A sample without RNA and a sample of t-RNA (10 μg) were used as negative controls. The primer extension products shown in Fig. 2 are indicated by an asterisk above the appropriate nucleotide of the indicated sequence. Starting points of the RACE products are indicated by an arrow underneath the sequence. The number of RACE clones (total 25) starting at a particular base is indicated by the number shown below the arrows. The site where 44% (11/25) RACE clones started was assigned +1.

Both methods demonstrated the existence of several transcription start sites. The PCR product from the RACE reaction consisted of a single band of ~450 bp. Sequencing of the inserts after cloning revealed that 80% of the RACE products (20/25) started from a 4 base pair stretch, and thus the predominant start site was assigned +1. This site is 130 bp upstream of the translation initiating ATG codon. Primer extension analysis identified the same start sites, but minor products were also seen further upstream (Fig. 2). The major start site coincides with the RACE results of the 5' end of the cDNA clone described by Yang *et al.* (1997). Neither RACE clones nor primer extension assays showed evidence for a second transcript in myeloid leukemia cells that could indicate a transcriptional start site upstream of the second ATG in intron 1 (data not shown).

Example 15: Potential transcription factor binding sites in the 5' upstream region.

Genomic sequences 1299 bp upstream of the transcription start site were cloned and sequenced. No TATA box was found in proximity to the putative transcriptional start site. The main transcriptional start site is likely to function as an initiator region (Inr) since the

Both the -1299 to +144 and the -190 to +144 constructs exhibited promoter activity in a variety of cell lines including Cos-7(monkey kidney cell), MCF-7 (breast cancer cell), U937 (myeloid leukemia cell), KCL22 (myeloid leukemia cell), PC3 (prostatic cancer cell), Hela (cervical cancer cell) and Jurkat (T-cell lymphoma). (Data not shown). In all of these mammalian cell lines, luciferase activities generated by the -190 to +144 construct were higher than those by the -1299 to +144 construct. Constructs with a 5' end containing less than 190 bp upstream of the transcription start site showed a progressive loss of promoter activity. A construct containing bp -37 to +144 showed only two-fold higher activity than the promoterless vector PGL3-Basic.

Example 17: Role of Sp1 and GC boxes for transcriptional activity of the cyclin A1 promoter.

TATA-less promoters frequently depend on GC boxes to activate transcription. (J. Lu *et al.*, J. Biol. Chem. 269:5391-5402 [1994]; M.C. Blake *et al.*, Molec. Cell. Biol. 10:6632-41 [1990]). One of the main classes of transcription factors binding to these sites are Sp1 family proteins (A.J. Courey and R. Tjian, Cell 55:887-98 [1988]; A.P. Kumar and A.P. Butler, Nucleic Acids Res. 25:2012-19 [1997]; G. Hagen et al., J. Biol. Chem. 270:24989-94 [1995]). The cyclin A1 promoter contains at least six potential GC boxes between 190 and 37 bp upstream of the transcription start site. The importance of Sp1 for the activity of the cyclin A1 promoter, was demonstrated by the use of various promoter constructs that were transfected into the *Drosophila* cell line S2, which lacks endogenous Sp1 and Sp3.

Figure 5 shows activity of the cyclin A1 promoter fragments in the *Drosophila* cell line S2. Activity is indicated as fold activation of PGL3-Basic as compared to reporter gene activity without addition of Sp1 expression plasmid. The punctated and solid bars represent activities without and with Sp1 co-expression, respectively. When transfected alone, the activity of all cyclin A1 promoter fragments was not significantly different from the empty vector control. (Fig. 5, dotted bars).

The addition of a Sp1 expression plasmid strongly activated transcription by 15- to 25-fold from the cyclin A1 promoter. (Fig. 5, solid bars). Increased transcriptional activity was observed only for constructs containing sequences starting between -1299 and -112 bp upstream of the transcription start site. The construct containing the nucleotide sequences between -37 and +144 did not show any increase in activity, implying that Sp1 binding sites

[1995]). To analyze cell cycle regulation of promoter activity, transiently transfected cells were arrested in different phases of the cell cycle and subsequently analyzed for luciferase activity. Cell cycle regulated activity was found for the full length promoter as well as for the construct containing the -190 to +144 fragment.

Figure 7 shows Cell cycle regulated activity of the cyclin A1 promoter in Hela cells. In Figure 7(A), Hela cells were cell cycle arrested after transfection with a luciferase construct containing nt -190 to +144 of the cyclin A1 promoter. Cells were subsequently analyzed for luciferase activity. Cell cycle synchronization was confirmed by flow cytometry (data not shown). The bars represent means and SEM of at least three independent experiments. Promoter activity at 0 h was set as 1. The cyclin A1 promoter activity was relatively low during the G_0/G_1 phase. It increased after the cell cycle progressed beyond the G_1/S boundary.

In Figure 7(B), Hela cells were synchronized at the G_1/S boundary using aphidicolin, following transient transfection and serum starvation. Cells were released from the block and harvested at the indicated time points for luciferase and cell cycle analyses. The graph depicts data from a representative experiment. When transiently transfected Hela cells were released from an aphidicolin block, luciferase values started to increase after 6 hours and reached a maximum after 12-16 h.

Figure 7(C) shows cell cycle distribution at the different time points of the time-release experiment. The hatched, open and solid bars represent G_1, S and G_2/M phases, respectively. The highest levels of activity were observed in the S and G_2/M phases. The maximum promoter activity corresponded to the percentage of cells present in the S and G_2/M phases. This is consistent with data showing that levels of cyclin A1 mRNA accumulate during S phase, with the highest levels present at the S and G_2/M phases. (Yang *et al.*, Mol. Cell. Biol. [in press 1999]).

Fragments containing nucleotides -1299 to +144, -190 to +144, or -190 to +13 performed similarly in all these experiments (data not shown).

Various point mutations and deletions were generated in the presumed E2F sites and the suspected CDE element in order to define the regions that are relevant for cell cycle regulation of the cyclin A1 promoter. Activity of the wild type construct (containing the -1299 to +144 fragment) in aphidicolin arrested cells was set as 1.0 and compared to the other constructs. Only a 40% decrease was detected for the construct containing the four mutated

tails. The mice were anesthetized with metafane. and a 1-cm piece of tail tip was cut using a sterile scalpel. The tail biopsy was incubated with 100μg of Proteinase K in 700μL lysis buffer (10 mM Tris, pH7.5, 1mM EDTA, and 10% SDS) overnight at 50°C. The lysate was extracted once with 500μl phenol, twice with phenol/chloroform (1:1) and was precipitated with ice cold isopropanol. The precipitate was centrifuged and the pellet was washed once with 70% ethanol. The pellet was allowed to air dry for 30 minutes at room temperature and was then resuspended in 200μL 10 mM Tris, pH 7.5, 0.1 mM EDTA. The tail DNA was allowed to incubate at 65°C for 10 min, and it was then stored at 4°C.

For each sample, 100 ng of tail DNA was added to a PCR cocktail mix in a total volume of 50μL. For each sample tube, the PCR cocktail contained 10μL of Qiagen Q buffer, 5μL of PCR buffer (Qiagen), dNTPs and a pair of EGFP-specific primers, 5'-TTG TCG GGC AGC AGC ACG GGG CCG-3' (SEQ. ID. NO.:30) and 5'-TCA CCG GGG TGG TGC CAT CCT TGG-3' (SEQ. ID. NO.:31). A 600 bp fragment was amplified. A positive control contained the cyclin A1-EGFP plasmid DNA, and a negative control contained no DNA.

Example 20: Selectable fluorescent vertebrate germ cells expressing EGFP by the cyclin A1 promoter

Five lines of transgenic mice were generated that contain DNA construct pCyclinA1-EGFP-1 and express the flourescent green reporter gene (EGFP) under the control of the cylin A1 promoter (cyclin A1-EGFP mice). Flourescent green protein is seen in male germ cells with FITC filter. The mice were transfected with a construct containing a 1.4 kb 5' flanking region DNA of human cyclin A1 including, nt. -1299 to +144, inserted into the *Bgl*II/*Hind*III site of the promoterless fluorescent green protein (EGFP) expression vector pEGFP-1 (Clontech; Figure 1). The vector also contained a SV40 splice and polyadenylation signal 3' to the EGFP gene, as well as kanamycin and neomycin resistance genes for selection purposes. The pCyclinA1-EGFP-1 construct was expressed in Cos-7, MCF-7, and U937 cells in vitro. For the generation of transgenic mice, the vector sequences were removed from the construct, and the DNA fragment which comprised the cyclin A1 promoter, the EGFP gene, and the SV40 splice and polyadenylation signal was purified on a 10%-40% sucrose gradient. One-milliliter fractions were collected from the gradient, and the fraction containing the construct

population; and populations 2 through 4, which had increasing fluorescence and scatter properties reflecting different cell types.

The cyclin A1-EGFP cells were also tested with PE conjugated PE anti-c-kit antibodies and analyzed with FACS. The FACS analysis showed that there is a population of fluorescent cells which expresses EGFP under the cyclin A1 promoter and that these cells are positive for c-kit. Some of the c-kit cells were not EGFP positive.

Figure 8 shows frozen sections from testis of adult mice that were cut, rinsed in phosphate buffered saline (PBS) for 10 min and analyzed by confocal laser scanning microscopy. Whereas no fluorescence could be observed in testicular tubuli of control mice (Fig. 8a), strong and highly specific expression of EGFP (Fig. 8b and c) was detected in testis of transgenic mice. Maximal EGFP expression was observed during and after the first meiotic division and a weaker staining was present in spermatogonia. Magnifications are 400x (Fig. 8a and b) and 100x (Fig. 8c).

Example 22: The effect of CpG methylation of the cyclin A1 promoter.

Bisulfite sequencing was carried out according to the method described by Clark *et al.* with minor modifications. (S.J. Clark et al., High sensitivity mapping of methylated cytosines. *Nucleic Acids Res* 22: 2990-2997 [1994]). Ten mg of DNA was incubated with the bisulfite/hydroquinone solution for six hours. A nested PCR was performed (detailed Primer information will be provided on request) and the final PCR product (ca. 400 bp) was gel purified. The PCR products were either blunt end cloned and at least 10 clones were sequenced, or the purified PCR product was directly sequenced using ^{33}P-cycle sequencing of nucleotides.

In vitro methylation and luciferase assay. The cyclin A1 promoter – luciferase reporter construct was *in vitro* methylated by SssI following the recommendations of the manufacturer (New England Biolabs). (S. Kudo, *Methyl-CpG binding protein MeCP2 represses Sp1-activated transcription of the human leukosialin gene when the promoter is methylated*, Mol. Cell. Biol.18:5492-99 [1998]). S2 Drosophila cells were transfected as described previously using 1 µg of methylated or mock-methylated luciferase – reporter plasmid, 100 ng of Sp1 expression plasmid and 1 µg of a CMV-β-galactosidase expression plasmid used for standardization purposes. One µg of human MeCP2 expression vector or empty vector control were co-

repression of transcriptional activity. (X. Nan et al. [1997] and [1998]). The human leukosialin gene is one of the genes shown to be negatively regulated by MeCP2 when its promoter is methylated. (S. Kudo [1998]). Leukosialin (similar to cyclin A1) is tissue-specifically expressed in hematopoietic cells and its transcriptional activity depends on the Sp1 transcription factor. Using S2 Drosophila cells that do not express endogenous MeCP2, it was analyzed whether co-transfected MeCP2 would suppress activity of the methylated (+) or unmethylated (-) cyclin A1 promoter (Fig. 9b). Upon transfection of *in vitro* methylated cyclin A1 promoter constructs into Drosophila cells, we noticed 3-fold repression without MeCP2. When MeCP2 was co-expressed with the methylated cyclin A1 promoter constructs, promoter activity was inhibited by 12-fold. indicating that MeCP2 can suppress transcriptional activation of the methylated cyclin A1 promoter. (Fig. 9b).

Since methylation appeared to be involved in regulation of the cyclin A1 gene in the mammalian cell lines, it was investigated whether the site of chromosomal integration would determine the patterns of methylation and expression of the transgenic cyclin A1 promoter. Four lines of transgenic mice carried the cyclin A1 promoter – EGFP reporter construct, as described above; this was the same nucleic acid construct used to generate the stable MG63 cell line. All lines of transgenic mice showed highly specific expression in the testis resembling the expression pattern previously determined by *in-situ* hybridization techniques. (Fig. 8; C. Sweeney *et al.* [1996]). The EGFP expression pattern in testis was indistinguishable among the different lines. The cyclin A1 promoter was able to direct tissue specific expression in the testis independent of the chromosomal integration site. The methylation status of a transgene is thought to be largely determined by either the chromatin structure at the site of integration, the *cis*-acting sequences in the transgene, and/or the influence of a locus control region. (J.R. Chaillet *et al.*, *Parental-specific methylation of imprinted transgene is established during gametogenesis and progressively changes during embryogenesis*, Cell 66:77-83 [1991]; K. Matsuo *et al.*, *An embryonic demethylation mechanism involving binding of transcription factors to replicating DNA*, EMBO J. 17:1446-53 [1998]; M. Brandeis *et al.*, Nature 371:435-38 [1994]). Transgene activity has also been reported to be associated with hypomethylation. (E.g., Pikaart *et al.* [1998]). Analysis of the methylation status of the human cyclin A1 promoter in the testis of four transgenic mouse lines showed that the promoter and the transgene were not methylated in the testis of two lines. However, the promoter and the transgene were heavily methylated in testis of the two other lines. No difference

-1299 to +144 and from -190 to +144 showed activity in all four cell lines (Fig. 10). The reporter activity of the shorter promoter fragment was always higher than the activity of the longer fragment. In addition, the activity of the cyclin A1 promoter was higher than that of the SV40 promoter (without enhancer) in all four cell lines.

The cyclin A2 promoter is tightly cell cycle regulated and is assumed to be transactivated in all cycling mammalian cells. Activity of the cyclin A2 promoter was detectable in all four cell lines, but the degree of activity was inversely correlated with the cyclin A1 promoter activity. Cyclin A2 promoter activity was higher in PC3 and Hela cells and it was lower in the myeloid cell lines as compared to the cyclin A1 promoter activity. (Fig. 10). Preferential activity of the cyclin A1 promoter in myeloid cells (compared to the cyclin A2 promoter) was evident for both promoter constructs tested. The inverse relationship between cyclin A2 and cyclin A1 was also present at the RNA level in samples from patients with acute myeloid leukemia. (R. Yang *et al.* [1999]). However, activity of the cyclin A1 promoter by transient transfection was not limited to the myeloid cell lines but was also present in PC3 and Hela cells. The tissues from which these cell lines derived express very low levels of cyclin A1. An explanation could be that transcription factors expressed in the cell lines, but not expressed in the normal tissue, lead to aberrant promoter activity. One transcription factor expressed in a wide variety of cell lines is c-myb. Western blot analysis demonstrated expression of c-myb in all four cell lines as well as in ML-1, another myeloid cell line that expresses high levels of cyclin A1. The non-myeloid cell lines appeared to have only a high molecular weight form while the myeloid lines had both a high and a low molecular weight form. This may reflect a phosphorylated and a non-phosphorylated myb protein.

antibody implying that c-myb either did not or only weakly bound this site. To test whether c-myb activation of the promoter was affected by alteration of the myb binding sites. different sites were mutated and the resulting constructs were transfected in KCL22 cells. These cells showed the highest c-myb expression of all the cell lines. Abrogation of the myb site at +2 clearly diminished promoter activity by 50% whereas a mutation at either -27 or mutation of the ets site at –15 did not lead to a decrease in promoter activity. The myb site at +2 to +5 is close to the transcriptional start site and the base pairs surrounding the transcriptional start site could function as an Initiator (Inr). To rule out that the observed effects of the mutation at +2 depended on the loss of binding of the basal transcriptional machinery, we transfected the mutated reporter construct together with the c-myb expression plasmid or an empty vector control into CV-1 cells and compared the results with transfections using the wildtype promoter plasmid. The mutation at +2 led to a minor reduction in promoter activity when transfected with the empty vector control. However, transactivation of the mutated reporter plasmid by c-myb was reduced by more than 50%, indicating that c-myb can transactivate the cyclin A1 promoter through this site. Other sites or indirect effects may contribute to the cyclin A1 promoter activation, because the mutation at +2 did not abolish the increase in promoter activity entirely. Different amounts of c-myb were co-expressed with a cyclin A1 promoter construct (-190 to +144 fragment). Empty vector was used to reach the same total amount of DNA in all experiments. Mean and standard error for three independent experiments are shown.

The foregoing examples being illustrative but not an exhaustive description of the embodiments of the present invention, the following claims are presented.

7. The method of Claim 1, wherein said fluorescent or light-emitting protein is a green fluorescent protein, yellow fluorescent protein, blue fluorescent protein, phycobiliprotein, luciferase, or apoaequorin.

8. The method of Claim 1, wherein said vertebrate is a mammal or bird.

9. The method of Claim 1, wherein said vertebrate is a human, non-human primate, mouse, rat, rabbit, gerbil, hamster, canine, feline, ovine, bovine, swine, pachyderm, equine, or a farm or marine mammal.

10. The method of Claim 1, wherein said vertebrate is a duck, chicken, goose, ostrich, emu, dove, quail, guinea fowl, or turkey.

11. The method of Claim 1, wherein said germ cell or precursor cell develops into a maturing male gamete after said polynucleotide is incorporated into the genome of said germ cell or precursor cell.

12. The method of Claim 2, wherein a stem cell of said progeny is grown in vitro.

13. The method of Claim 12, wherein said stem cell is grown in the presence of an inhibitor of DNA methylation.

14. A selectable transgenic stem cell obtained by the method of Claim 1.

15. The selectable transgenic stem cell of Claim 14, wherein said stem cell is a pluripotent, multipotent, bipotent, or monopotent stem cell.

16. The selectable transgenic stem cell of Claim 14, wherein said stem cell is a spermatogonial, embryonic, osteogenic, hematopoietic, granulopoietic, sympathoadrenal, mesenchymal, epidermal, neuronal, neural crest, O-2A progenitor, brain, kidney, pancreatic, liver or cardiac stem cell.

17. The selectable transgenic stem cell of Claim 14, wherein said stem cell is a selectable transgenic male germ cell.

24. The method of Claim 23, wherein breeding is by in vitro or in vivo fertilization of an ovum of said female.

25. The method of Claim 22, wherein said cyclin A1 promoter sequence comprises SEQ. ID. NO.:2, or an operative fragment or non-human homologue thereof, or an operative derivative of any of these.

26. The method of Claim 22, wherein said polynucleotide further comprises at least one insulator element flanking said transcriptional unit, whereby methylation in vivo of said promoter sequence is substantially prevented.

27. The method of Claim 26, wherein at least one of said insulator element(s) is a chicken β-globin insulator element.

28. The method of Claim 22, wherein said fluorescent or light-emitting protein is a green fluorescent protein, yellow fluorescent protein, blue fluorescent protein, phycobiliprotein, luciferase or apoaequorin.

29. The method of Claim 22, wherein said vertebrate is a mammal or bird.

30. The method of Claim 22, wherein said vertebrate is a human, non-human primate, mouse, rat, rabbit, gerbil, hamster, canine, feline, ovine, bovine, swine, pachyderm, equine, or a farm or marine mammal.

31. The method of Claim 22, wherein said vertebrate is a duck, chicken, goose, ostrich, emu, dove, quail, guinea fowl, or turkey.

32. The method of Claim 22, wherein said germ cell or precursor cell develops into a maturing male gamete after said polynucleotide is incorporated into the genome of said germ cell or precursor cell.

33. The method of Claim 23, wherein a stem cell of said progeny is grown in vitro.

administering to a gonad of a male vertebrate a transfection mixture comprising at least one transfecting agent and at least one polynucleotide comprising a transcriptional unit of a cyclin A1 promoter sequence operatively linked to a DNA encoding a fluorescent or light-emitting protein, under conditions effective to reach a germ cell or germ cell precursor of the male vertebrate; and

causing said polynucleotide to be taken up by, and released into, said germ cell or precursor cell;

incorporating said polynucleotide into the genome of said germ cell or precursor cell;

allowing said germ cell or precursor cell to develop into a maturing male gamete; and

breeding said male vertebrate with a female of its species to obtain a transgenic progeny expressing said fluorescent or light-emitting protein in at least one of its stem cells, whereby said stem cell can be isolated or selected from a non-stem cell.

44. The method of Claim 43, wherein breeding is by in vitro or in vivo fertilization of an ovum of said female.

45. The method of Claim 43, wherein said cyclin A1 promoter sequence comprises SEQ. ID. NO.:2, or an operative fragment or non-human homologue thereof, or an operative derivative of any of these.

46. The method of Claim 43, wherein said polynucleotide further comprises at least one insulator element flanking said transcriptional unit, whereby methylation in vivo of said promoter sequence is substantially prevented.

47. The method of Claim 46, wherein at least one of said insulator element(s) is a chicken β-globin insulator element.

48. The method of Claim 43, wherein said fluorescent or light-emitting protein is a green fluorescent protein, yellow fluorescent protein, blue fluorescent protein, phycobiliprotein, luciferase or apoaequorin.

49. The method of Claim 43, wherein said vertebrate is a mammal or bird.

62. A method of producing a non-human transgenic vertebrate animal line having native

germ cells, comprising

breeding the vertebrate of Claim 58 with a member of the opposite sex of the same species; and selecting progeny for stem cell-specific expression of a xenogeneic fluorescent or light-emitting protein.

63. A method of obtaining a selectable stem cell, comprising:

obtaining a maturing male germ cell from a vertebrate;

transfecting said male germ cell in vitro with at least one polynucleotide comprising a transcriptional unit of a stem cell-specific promoter operatively linked to a DNA encoding a fluorescent or light-emitting protein, in the presence of a gene delivery mixture comprising at least one transfecting agent, at about or below the vertebrate's body temperature and for a transfection-effective period of time;

causing said polynucleotide to be taken up by, and released into said germ cell; and

fertilizing an ovum with said germ cell such that a transgenic progeny expressing said fluorescent or light-emitting protein in at least one of its stem cells is obtained, said stem cell(s) being selectable from non-stem cells by detecting light emissions from said stem cell(s).

64. The method of Claim 63, wherein fertilizing an ovum is by in vitro or in vivo fertilization.

65. The method of Claim 63, wherein said stem cell-specific promoter is a cyclin A1 promoter.

66. The method of Claim 63, wherein said cyclin A1 promoter sequence comprises SEQ. ID. NO.:2, or an operative fragment or non-human homologue thereof, or an operative derivative of any of these.

67. The method of Claim 63, wherein said polynucleotide further comprises at least one insulator element flanking said transcriptional unit, whereby methylation in vivo of said promoter sequence is substantially prevented.

79. A transgenic non-human vertebrate comprising the selectable transgenic stem cell of Claim 75.

80. The transgenic non-human vertebrate of Claim 79, wherein said vertebrate is a non-human mammal or a bird.

81. Vertebrate semen comprising the male germ cell of Claim 78.

82. A method of producing a non-human transgenic vertebrate animal line having native germ cells, comprising

breeding the vertebrate of Claim 79 with a member of the opposite sex of the same species; and selecting progeny for stem cell-specific expression of a xenogeneic fluorescent or light-emitting protein.

83. A method of obtaining a selectable stem cell, comprising:

obtaining a maturing male germ cell from a vertebrate;

transfecting said male germ cell in vitro with at least one polynucleotide comprising a transcriptional unit of a cyclin A1 promoter operatively linked to a DNA encoding a fluorescent or light-emitting protein, in the presence of a gene delivery mixture comprising at least one transfecting agent, at about or below the vertebrate's body temperature and for a transfection-effective period of time; and

allowing said polynucleotide to be taken up by, and released into said germ cell;

fertilizing an ovum with said germ cell such that a transgenic progeny expressing said fluorescent or light-emitting protein in at least one of its stem cells is obtained, said stem cell(s) being selectable from non-stem cells by detecting light emissions from said stem cell(s).

84. The method of Claim 83, wherein fertilizing an ovum is by in vitro or in vivo fertilization.

85. The method of Claim 83, wherein said cyclin A1 promoter sequence comprises SEQ. ID. NO.:2, or an operative fragment or non-human homologue thereof, or an operative derivative of any of these.

mesenchymal, epidermal, neuronal, neural crest, O-2A progenitor, brain, kidney, pancreatic, liver or cardiac stem cell.

97. The selectable transgenic stem cell of Claim 94, wherein said stem cell is a selectable transgenic female or male germ cell.

98. A transgenic non-human vertebrate comprising the stem cell of Claim 94.

99. The transgenic non-human vertebrate of Claim 98, wherein said vertebrate is a non-human mammal or a bird.

100. Vertebrate semen comprising the male germ cell of Claim 97.

101. A method of producing a non-human transgenic vertebrate animal line having native germ cells, comprising

breeding of the vertebrate of Claim 98 with a member of the opposite sex of the same species; and selecting progeny for stem cell-specific expression of a xenogeneic fluorescent or light–emitting protein.

102. A nucleic acid construct, comprising a cyclin A1 promoter having nucleotide sequence (SEQ. ID. NO.:2), or an operative fragment or non-human homologue thereof, or an operative derivative of any of these.

103. The nucleic acid construct of Claim 102, further comprising said cyclin A1 promoter operatively linked to a nucleotide sequence encoding a fluorescent or light-emitting protein, as a transcriptional unit.

104. The nucleic acid construct of Claim 103, wherein said polynucleotide further comprises at least one insulator element flanking said transcriptional unit.

105. The nucleic acid construct of Claim 104, wherein at least one of said insulator element(s) is a chicken β-globin insulator element.

117. The transgenic non-human vertebrate of Claim 115, wherein said vertebrate is a duck, chicken, goose, ostrich, emu, dove, quail, guinea fowl, or turkey.

118 A kit for transfecting a male vertebrate's germ cells, comprising:

a transfecting agent and a polynucleotide comprising a transcriptional unit of a human cyclin A1 promoter sequence having SEQ. ID. NO.:2, or an operative fragment or non-human homologue thereof, or an operative derivative of any of these, operatively linked to a DNA having a nucleotide sequence encoding a fluorescent or light-emitting protein, whereby said kit may be used to transfect said germ cells.

119. The kit of Claim 118, wherein the transfecting agent is a liposome, viral vector, transferrin-polylysine enhanced viral vector, retroviral vector, lentiviral vector, or uptake enhancing DNA segment, or a mixture of any of these.

120. The kit of Claim 118, wherein the transfecting agent comprises a retroviral vector, adenoviral vector, transferrin-polylysine enhanced adenoviral vector, human immunodeficiency virus vector, lentiviral vector, Moloney murine leukemia virus-derived vector, mumps vector, a DNA segment that facilitates polynucleotide uptake by and release into the cytoplasm of germ cells, or comprises an operative fragment of- or mixture of any of these.

121. The kit of Claim 118, wherein the transfecting agent comprises an adenovirus vector having endosomal lytic activity, and the polynucleotide is operatively linked to the vector.

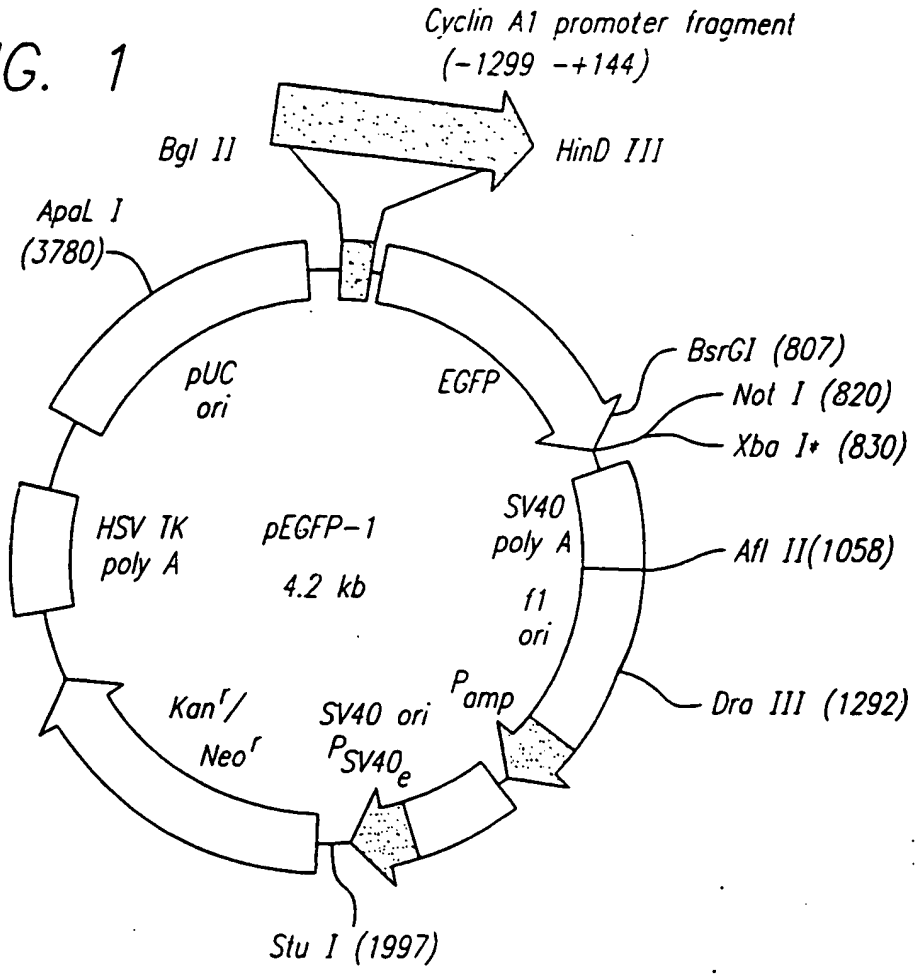
122. The kit of Claim 118, wherein the transfecting agent comprises a lipid transfecting agent.

123. The kit of Claim 118, wherein the transfecting agent further comprises a male-germ-cell-targeting molecule.

124. The kit of Claim 123, wherein the male-germ-cell-targeting molecule is specific for targeting spermatogonia and comprises a c-kit ligand.

125. The kit of Claim 118, further comprising an immunosuppressing agent.

FIG. 1



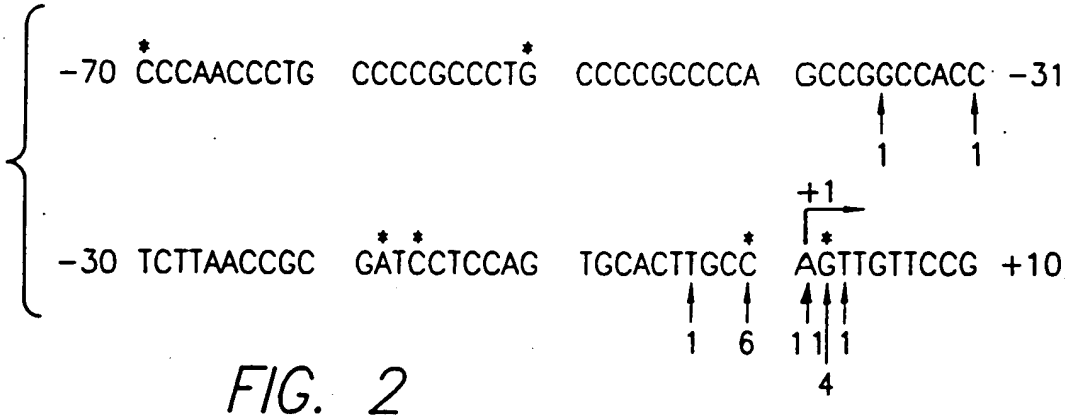
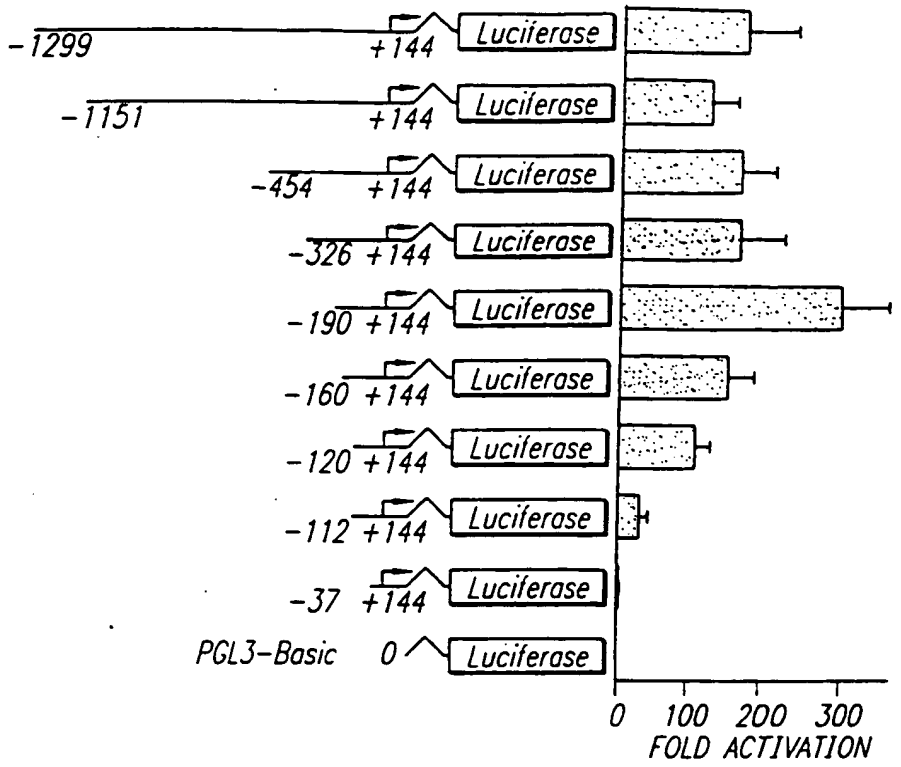


FIG. 2

FIG. 4



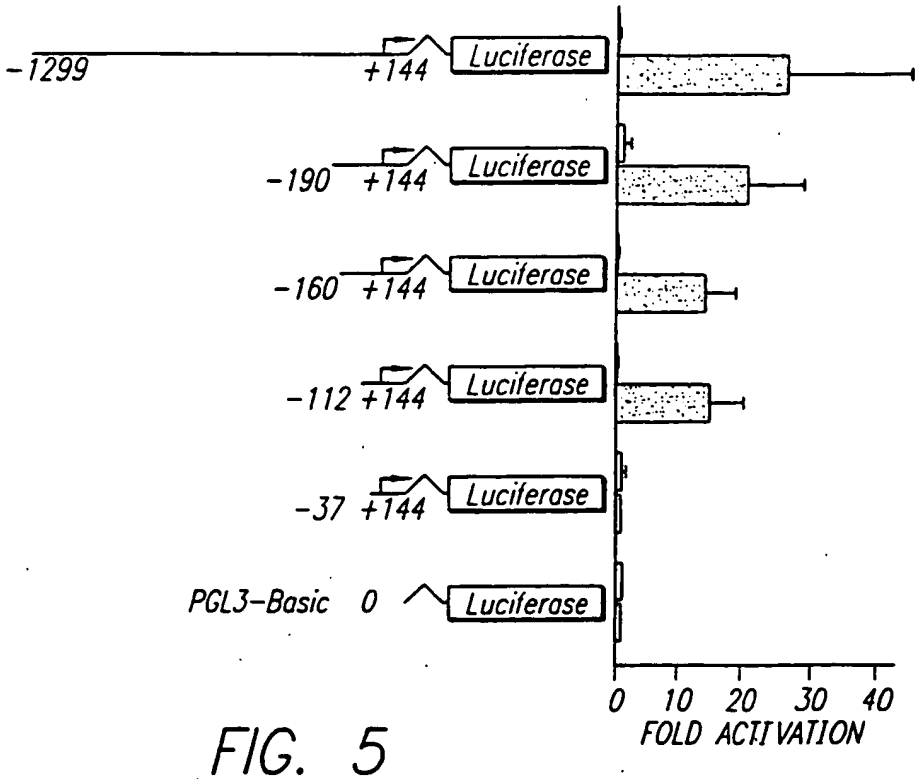


FIG. 5

FIG. 7a

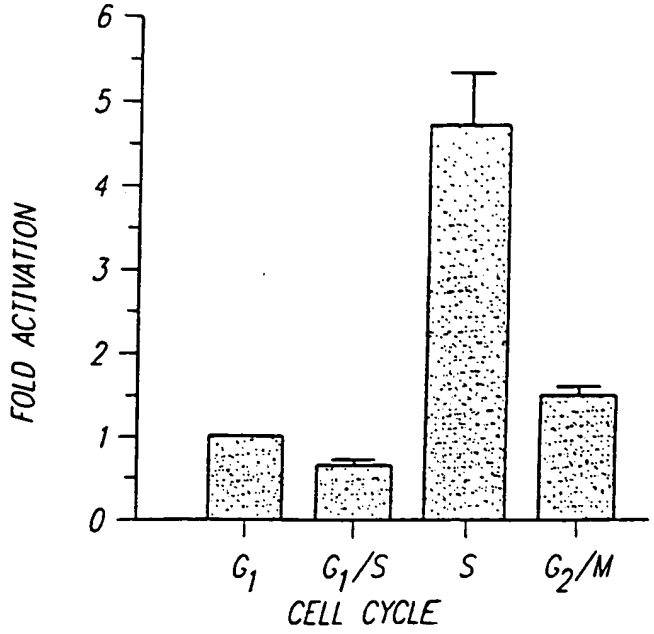


FIG. 7b

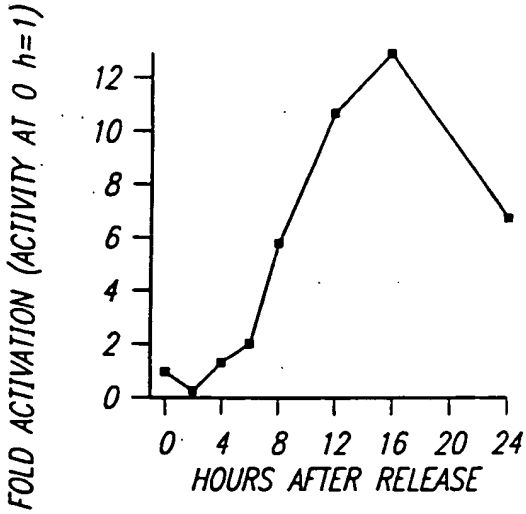


FIG. 7c

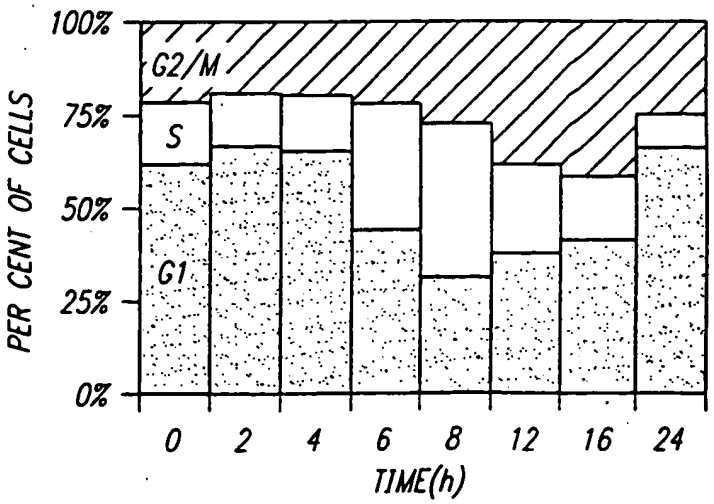


FIG. 9b

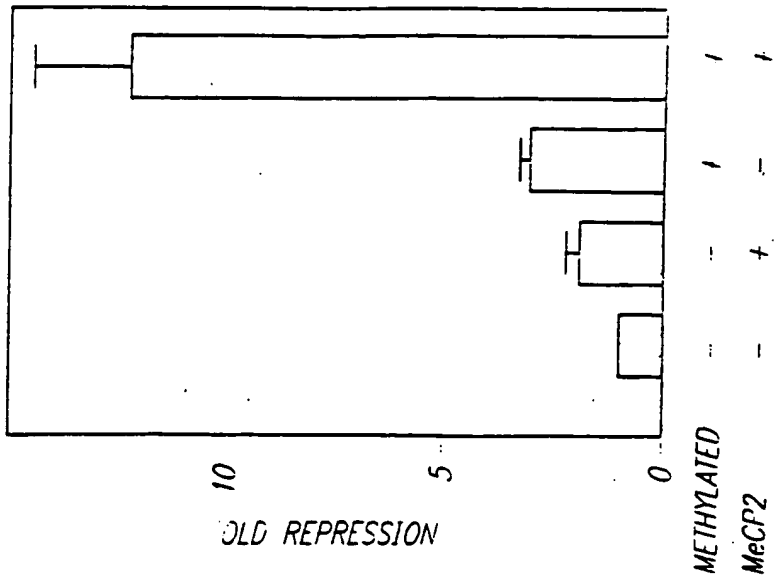


FIG. 9a

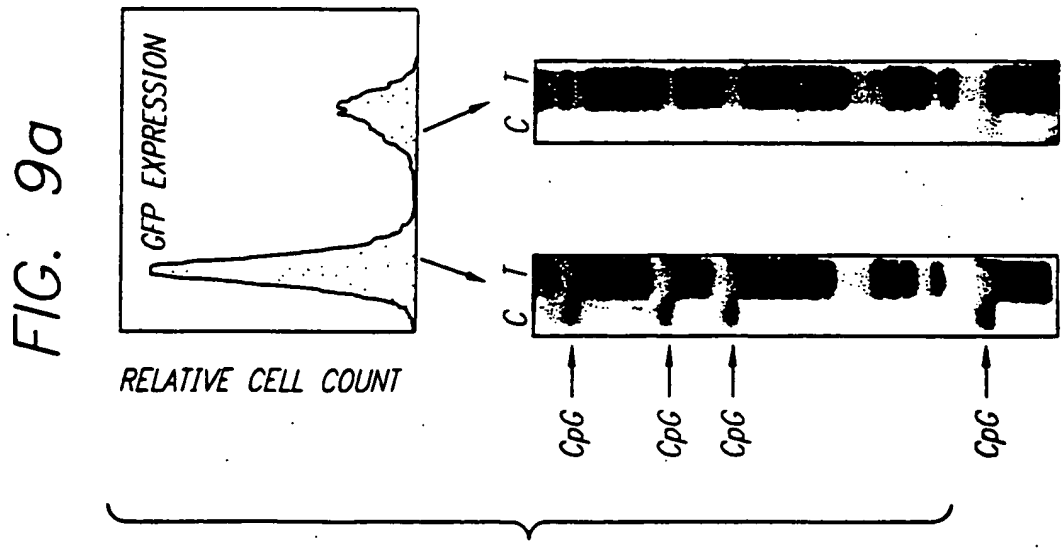


FIG. 11b

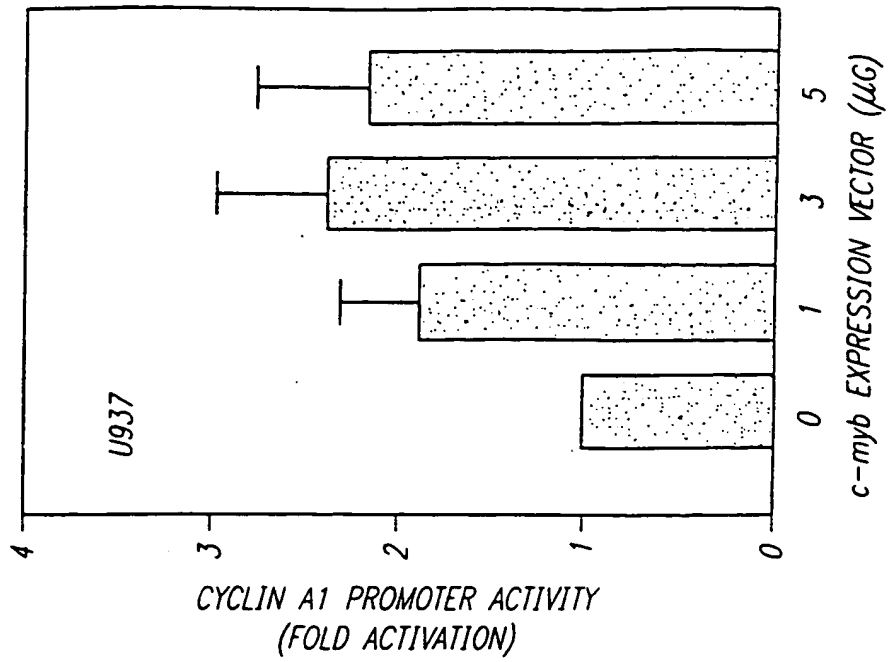
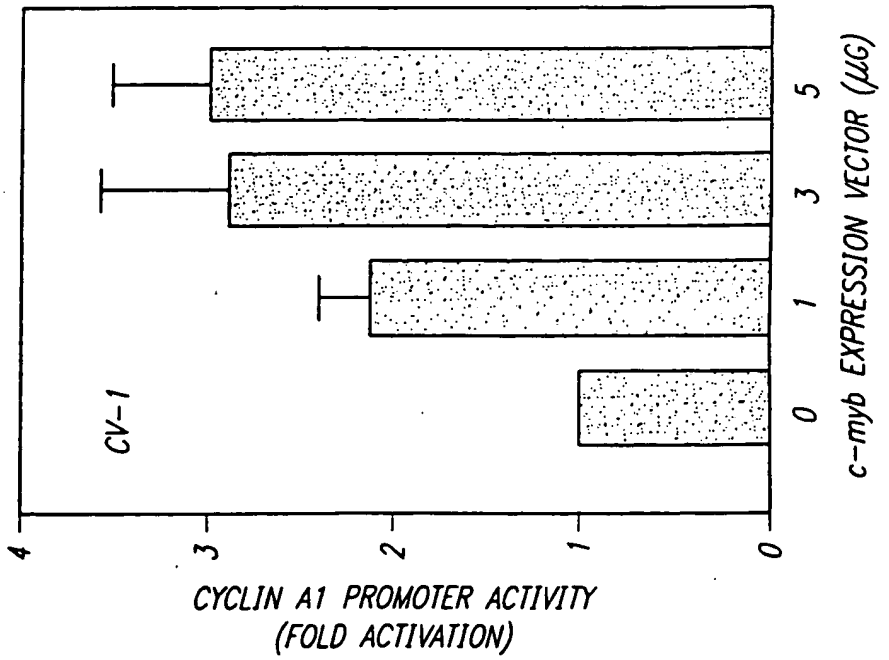


FIG. 11a



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<223> EGFP-specific single-stranded oligonucleotide

<400> 31

INTERNATIONAL SEARCH REPORT

International Application No
PCT/US 99/08277

A. CLASSIFICATION OF SUBJECT MATTER
IPC 7 C12N15/89 C12N15/86 C12N15/88 C12N5/06 A01K67/027
A61K48/00 C12N15/65 C07K14/47 C12Q1/68

According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)
IPC 7 C12N A01K C07K C12Q

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practical, search terms used)

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category *	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	MURAMATSU T ET AL: "Foreign gene expression in the mouse testis by localized in vivo gene transfer" BIOCHEMICAL AND BIOPHYSICAL RESEARCH COMMUNICATIONS., vol. 233, no. 1, 7 April 1997 (1997-04-07), pages 45-49, XP002099972 ORLANDO, FL US the whole document	1,7-9, 14-19, 115,127, 129
X	EP 0 867 114 A (HOECHST MARION ROUSSEL LTD (JP)) 30 September 1998 (1998-09-30)	14-21, 35-42, 54-62
A	the whole document	18,19, 119,120, 122
Y		1,22,43

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[X] Further documents are listed in the continuation of box C.

[X] Patent family members are listed in annex.

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"&" document member of the same patent family

Date of the actual completion of the international search	Date of mailing of the international search report
4 October 1999	15/10/1999

Name and mailing address of the ISA	Authorized officer
European Patent Office, P.B. 5818 Patentlaan 2 NL - 2280 HV Rijswijk Tel. (+31-70) 340-2040. Tx. 31 651 epo nl. Fax: (+31-70) 340-3016	De Kok, A

Form PCT/ISA/210 (second sheet) (July 1992)

C.(Continuation) DOCUMENTS CONSIDERED TO BE RELEVANT

Category	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No
A	WO 92 03459 A (SLOAN KETTERING INST CANCER) 5 March 1992 (1992-03-05) page 1, line 1 -page 7, line 12 page 27, line 21 - line 28	123,124
A	YANG R ET AL.: "Characterization of a second human cyclin A that is highly expressed in testis and in several leukemic cell lines" CANCER RESEARCH., vol. 57, 1997, pages 913-920, XP002115937 AMERICAN ASSOCIATION FOR CANCER RESEARCH, BALTIMORE, MD., US ISSN: 0008-5472 the whole document	4,25,45, 55,102
A	WO 94 23046 A (US HEALTH) 13 October 1994 (1994-10-13) the whole document	1,5,6, 16,17, 46,47, 67,68, 104,105
A	IKAWA M ET AL: "Green fluorescent protein as a marker in transgenic mice" DEVELOPMENT GROWTH AND DIFFERENTIATION, vol. 37, 1 August 1995 (1995-08-01), pages 455-459, XP002086829 ISSN: 0012-1592 the whole document	1

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